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This research and development project entitled "Nerve Agent Sensing Biopolymer Wipe" is directed at developing a simple-to-use enzyme-containing sensor for detecting nerve agent contamination at surfaces, in air and in solution, and to provide a tool for early and accurate identification of the chemical agents. The objective of this research effort consists of presenting a prototype to the end-user community, which is highly sensitive, easy to use, and accurate. The Agentase nerve agent outperforms any conventional technology for nerve agent detection in its simplicity of use, interference resistance, broad-based compatibility with surfaces, liquids, and gases, and low cost. The sensor is self-contained and simply pressed against a surface to initiate the reaction equilibrium described above. The substrate and enzymes are also each directly integrated within the polymer layers to remove any requirement for applying additional substrates or extended incubation times, as is the case with conventional technologies. Similar to a traffic light, the yellow sensor develops a red color after exposure to a contaminated surface and a green color to indicate a clean surface. In the existing prototype, red color is developed in less than 2 minutes, while the full green color development takes roughly 20 minutes.

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Nerve Agent Sensing Biopolymer Wipe

Final Report
April, 2003

by

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Statement of the problem studied

This research and development project entitled "Nerve Agent Sensing Biopolymer Wipe" is directed at developing a simple-to-use enzyme-containing sensor for detecting nerve agent contamination at surfaces, in air and in solution, and to provide a tool for early and accurate identification of the chemical agents. Recent events have illustrated that the general population is under constant threat of chemical warfare attack, and that terrorist organizations have the means, know-how, and intent to use chemical and biological weapons against civilian population. Rapid agent detection can initiate a situational response (whether it evacuation, wearing protective equipment, or triggering properly trained response teams) that limits exposure. Proper identification of agents ensures that subsequent medical and decontamination treatments are appropriate. Early warning and continuous monitoring equipment is of urgent need. This research project addresses the need for an inexpensive, simple to use single point sensor for the detection of nerve agents.

There are many products that can identify chemical weapons. They range in complexity from simple colormetric chemical test strips or gas tubes, to more complex multi-step test kits, to intricate arrays of gas chromatographs and mass spectrometers. Each method of agent identification has its own set of attributes and limitations. Tubes and test strips are simple to use and inexpensive but not precise and highly susceptible to interference. Multi-step test kits are more sensitive than simple test strips, but require intricate use protocols and remain susceptible to many forms of interference. More elaborate detection schemes such as the Chemical Agent Monitor (CAM) are more sensitive toward and resistant to some forms of interference, but are too expensive to use in large numbers. The Agentase nerve agent outperforms any conventional technology for nerve agent detection in its simplicity of use, interference resistance, broad-based compatibility with surfaces, liquids, and gases, and low cost.

The objective of this research effort consists of presenting a prototype to the end-user community, which is highly sensitive, easy to use, and accurate. This project shall eventually include removal of any requirement for applying a developing solution, the optimization of sensor formulation to achieve maximum performance and long-term stability, addressing potential environmental interference of the sensor and verification of compatibility with biological tissues.

Summary of the most important results

Introduction

Agentase has developed during this project entitled "Nerve Agent Sensing Biopolymer Wipe" and is currently in the process of marketing an enzyme-based biosensor capable of detecting nerve agents at surfaces, in solution and in air. The sensor technology makes use of the pH-dependent catalytic activity of enzymes to develop a dynamic pH equilibrium between two competing enzyme reactions. BChE catalyzed butyrylcholine hydrolysis results in the production of acid (decreasing pH) while urease-catalyzed urea hydrolysis produces base (increasing pH). Because of their relative positions on a pH dependence plot and the pH change each reaction induces, when both enzyme systems are active, an equilibrium is established between the two reactions, maintaining a constant pH. When agent is present, cholinesterase is inhibited and hydroxide ion production from urea hydrolysis drives a rapid increase in pH. The intellectual property and concepts behind this approach have been protected via an Agentase patent application entitled "Positive Response Biosensors and Other Sensors" (1).

Agentase's nerve agent sensors are hand-held devices consisting of two specially formulated polymers within an engineered applicator device. The intellectual property upon which the sensor and its design are based have been protected by one issued patent, US Patent #6,291,200 (2), and a submitted application entitled "Sensors for the Detection of an Analyte" (3). Key advancements over conventional nerve agent-sensing technology include simple protocols, broad-based compatibility, intuitive response, and resistance to common interferants. The sensor is self-contained and simply pressed against a surface to initiate the reaction equilibrium described above. The substrate and enzymes are also each directly integrated within the polymer layers to remove any requirement for applying additional substrates or extended incubation times, as is the case with conventional technologies. The enzyme polymer contains a co-immobilized pH sensitive indicator that transitions from yellow to red as the pH increases from 7 to 9. An additional cholinesterase substrate, indoxyl acetate, is included in the substrate polymer. Indoxyl acetate hydrolysis results in the production of blue indigo, providing the sensor with a mode of verifying performance and better signal differentiation. Similar to a traffic light, the yellow sensor develops a red color after exposure to a contaminated surface and a green color to indicate a clean surface. In the existing prototype, red color is developed in less than 2 minutes, while the full green color development exhibited in the photograph takes roughly 20 minutes. The current detection limit when using these protocols on diisopropyl fluorophosphate (DFP) is less than 100 ppb. Detection limit will vary for different agents based upon their ability to inhibit cholinesterase. Highly toxic materials such as warfare grade agents will be detected at even lower concentration levels.

While Agentase conducted sensor design and use protocol optimization using simulants in our laboratory, numerous studies have been conducted to demonstrate that simulant results correlate favorably with those using live agents. Agentase has taken part in live agent studies in the US, France, and the UK, including work at Dugway

Proving Ground and Edgewood. The Traffic Light Sensor has been operationally tested by a third party in the Human Intelligence and Counterintelligence Support Tools ACTD at the West Desert Test Facility in Dugway Proving Ground, Utah on May 23-24, 2001. The exercise was carried out by the Air Force Operational Test and Evaluation Center to discern the suitability, effectiveness, and overall utility of the Traffic Light Sensor. End-user feedback, third-party observations, and laboratory analyses were considered in the analysis. The Traffic Light Sensor received the highest achievable score, demonstrating utility and being recommended for immediate deployment *(4)*. Further operational assessments are presently underway with the 3rd Marine Division, 4th Marine regiment, Okinawa, Japan, US Naval Forces Central Command, Bahrain, and the US Embassy (4th Marine Expeditionary Brigade - FP/AT), Kabul, Afghanistan.

In summary, the Agentase development of the nerve agent sensor has been a major success. The sensor has progressed from a concept phase through proof-of-concept and prototype stages to a pre-production status in less than 2 years. Some important highlights of the development include effective manufacturing scale-up of synthesis chemistries, Beta-test agreements being established with federal agencies and domestic hospitals, presentation of the sensor to numerous potential interested parties including the BG Nilo and LTC Serino at the US Army Chemical School at Fort Leonard Wood, MO. Agentase has also taken substantial steps to ensure effective commercialization of the developed product. We are in the final stages of negotiating with a large manufacturer and distributor of equipment to emergency first responders. This relationship has provided Agentase with feedback from 86 end-user out of the domestic preparedness and homeland defense communities who ranked Agentases sensor very highly against other products in the marketplace. When asked if they were aware of any product similar to this detector, 87% responded no and more than 73% responded they were extremely interested in using the product to meet their job requirements.

Methods

Described below is a typical procedure for biopolymer synthesis. Variations of the reaction conditions affect both the physical properties of the resultant foam as well as the degree of enzyme-foam interaction. Initially 4 ml of buffer containing surfactant, pH sensitive dye and enzymes of interest are placed into a narrow cylindrical mixing vessel, prior to adding approximately 4 ml of prepolymer to the mixture. This 2 phase system is mixed with an in-house designed metal mixing head attached to a 2500 rpm hand held drill for 20 seconds. During the initial "cream" period, the solution is injected into a cylindrical mold where it rises and sets within 2 to 5 minutes. Polymer synthesis is complete in less than 10 minutes. The CO_2 evolved during the reaction of water and isocyanate lifts the foam to a final volume of approximately 50 to 60 ml. Surfactant selection varies CO_2 evolution and has significant effect on the porosity, density, and surface properties of resultant polymers. For the synthesis of substrate polymers, substrates are added to the buffer/ surfactant solution instead of pH dye and enzymes *(5-9).*

We used diisopropyl fluorophosphate (DFP) as a model for nerve agents in our standard sensing application. DFP dissolved in tap water was added either directly to the moistened sensor (solution) or onto a 70 cm^2 glass petri dish (surface). The signal, an obvious color change, could be monitored immediately after adding the analyte solution or wiping the test surface with the wet biopolymer wipe. In control reactions, fresh tap water was either added to the polymer sensor or onto the glass dish.

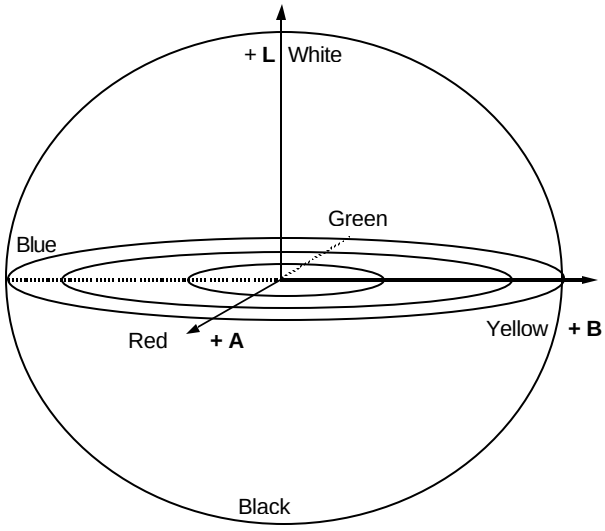

Figure 1. Representation of solid spectrophotometer 3-D color space.

One can follow the sensor signal by simply observing the color change under natural light with nothing more than the eye. In order to remove any subjectivity from our experimental procedures, we utilized a solid-phase Minolta CM-500d spectrophotometer to monitor the color change of the biocatalytic polymers. This unit uses a three-dimensional color coordinate system (L*a*b) to define colors and intensity (See Figure

1). Any visible color is defined within the coordinate system as a set of three positions. A color change is simply defined as the rate at which a position changes within the coordinate system. Figure 2 shows the color development after wiping a clean surface and a surface with nerve agent present. The positive a-axis describes the red color intensity and the negative a-axis describes the green color intensity. Both reactions the rapid development of red color and the slow development of the green color can be seen clearly by the spectrophotometer. This quantitative analysis of the color development has been the main tool in the initiated optimization of the sensor to achieve maximum performance.

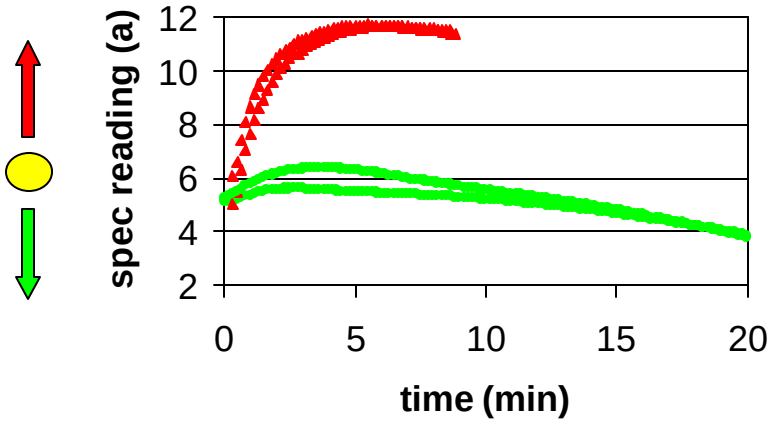

Figure 2. Signal development as monitored by spectrophotometer

Theoretical background

Agentase's technology for detecting nerve agents utilizes a second enzyme reaction to make signal development become more intuitive. We are synthesizing polyurethane polymers that contain both active butyryl cholinesterase (BChE) and urease enzymes. Rather than supplying only cholinesterase substrates to develop a color signal, ChE and urea are jointly provided to the polymer. Hydroxide ions resulting from the formation of ammonium during urease-catalyzed urea hydrolysis neutralize the protons produced during hydrolysis of cholinesterase substrate (butyryl choline). When nerve agents are absent, both enzyme systems (see Figure 4) are active, establishing an equilibrium between the two reactions and maintaining a constant pH. When an agent is present, hydroxide ions from urea hydrolysis are not neutralized because butyryl cholinesterase is inhibited. The pH of the sensor then rises, resulting in a positive signal (Figure 5). This construct provides a more intuitive response in that ***color change occurs in the presence of agent*** (or cholinesterase inhibition).

In order to fully understand the interactions between substrates, enzymes and pH-indicating dye it is important to analyze the reaction from the theoretical point of view. Enzyme activity is very often a function of pH, displaying a bell shape curve when plotted against pH with a pronounced pH optimum. This optimum is reported to be

around a pH of 8.0 for cholinesterase and near pH 7.0 for urease, the two enzymes utilized in the enzymatic sensor for nerve agents. BChE yields protons driving the pH down, while urease yields hydoxide ions driving the pH up. At the interception shown in Figure 3, both enzymes display the same activity resulting in an equilibrium where protons are neutralized by hydroxyl ions. This equilibrium will be somewhere in between the pH optimum of both enzymes. Figure 3 is an idealized schematic, with generalized pH dependence curves for both enzymes. By changing the ratio of BChE to urease this equilibrium can be swift to the left or the right.

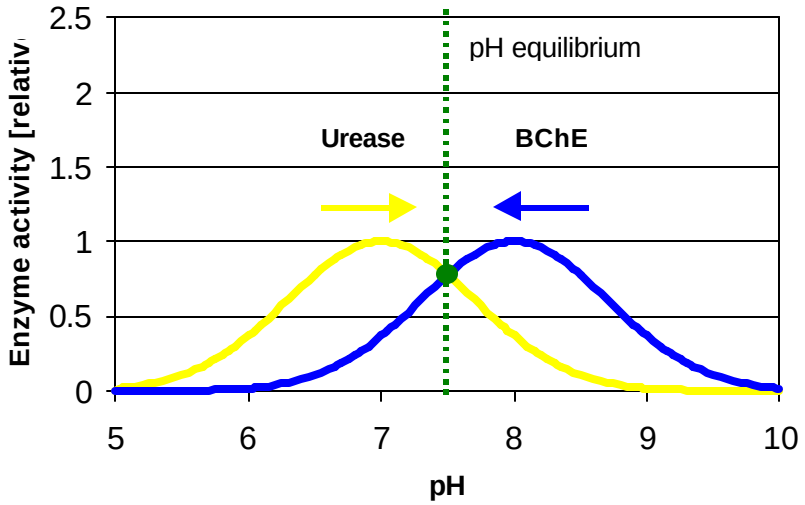

Figure 3. Urease and BChE (at two concentrations) activity as function of pH.

The sensor signal may be further improved by including an additional color-producing reaction to achieve a two-way color change. This two-way color change allows a positive response with two different colors in both the presence and absence of nerve agent. The reaction scheme illustrated in Figure 4 illustrates a two-way color change system in the detection of nerve agents. When nerve agents are present, the color changes from the yellow to red as a result of inhibition of cholinesterase and a corresponding pH increase. We have included indoxyl acetate as a third substrate within the system. When exposed to a clean surface (that is, the absence of nerve agents), cholinesterase hydrolyzes both butyrylcholine and indoxyl acetate. Hydrolyzed indoxyl acetate results in the formation of blue indigo. pH maintenance (resulting in the pH indicator remaining yellow) and the formation of blue indigo causes a color transition to green, resulting in a self-explanatory signal for both the contaminated and clean surfaces (See Figure 5).

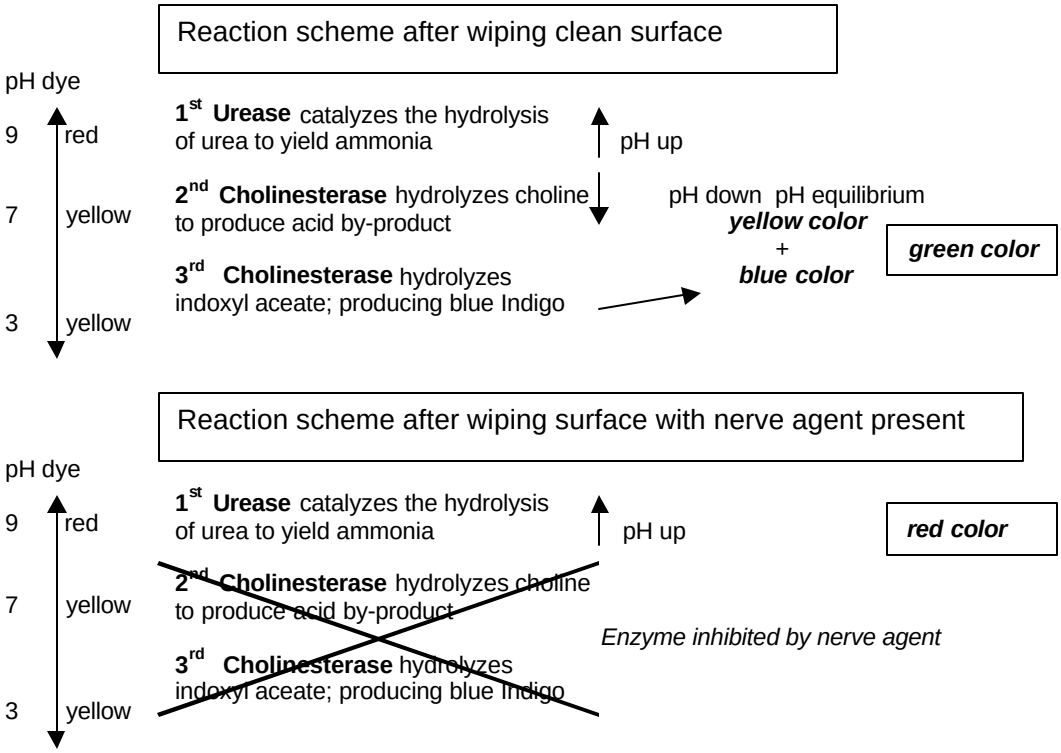

Figure 4. Reaction scheme Agentase nerve agent sensor

Figure 5 illustrates the straightforward-to-read signal exhibited by this sensor construct. Similar to a traffic light, the yellow sensor develops a red color after exposure to a contaminated surface and a green color to indicate a clean surface. In the existing prototype, red color is developed in less than 2 minutes, while the full green color development exhibited in the photograph takes roughly 20 minutes to 30 minutes. The color development of the green signal, which indicates the full functionality of the sensor as an added feature, needs to be delayed in order to avoid misinterpretation of the signal. It would be potentially difficult to distinguish between red and green since both signals emerge by darkening the initial yellow color of the sensing polymer, especially in the first 30 seconds.

after wiping contaminated surface after wiping clean (control) surface

Figure 5. Signal development in Agentase's Traffic Light Sensor Construct.

Discussion of components

The Agentase TL sensor contains two enzymes, three substrates and up to two different pH-indicating dyes. Each of these compounds has a pronounced effect upon sensor performance and sensitivity and will be discussed in detail.

Effect of enzyme ratio and concentration

As explained above, the relative concentrations of the enzymes employed is the most important factor in establishing equilibrium between the two enzyme reactions in the presence of adequate amounts of both substrates. To study the effect of the enzyme ratio on the pH equilibrium and to determine the best ratio for the initial sensor preparation, we added enzymes with varying ratio to a substrate solution and monitored the pH. The reactions started at a pH of 7.5 and the equilibrium were reached within two minutes. Keeping the BChE concentration constant we reduced the urease concentration down to a ratio of 200 to 1 (weight). The control reaction with no urease present resulted in a falling pH, as expected (from 7.5 down to 6.5 within 6minutes). The data points in Figure 6 (circles) show that urease dominated the soluble reaction system resulting in a high equilibrium pH. Considering the optimum at a pH around 7, the soluble urease seems to display high activity over a broad range of pH values.

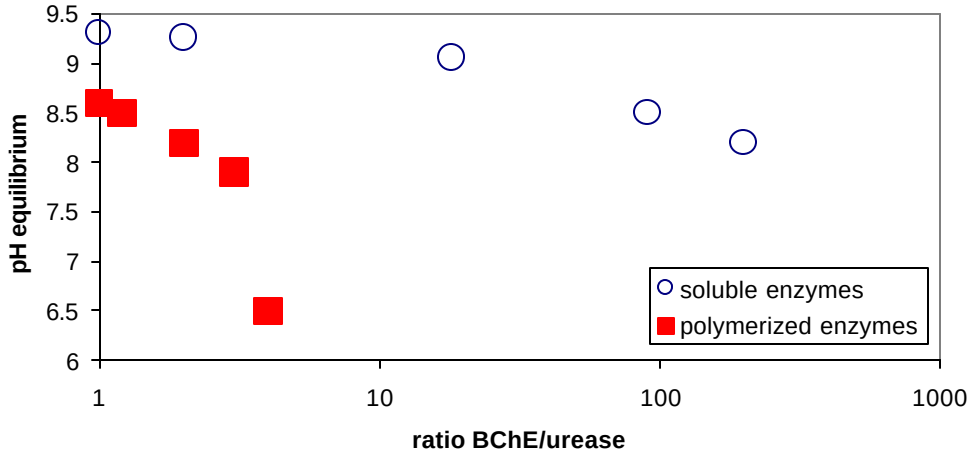

Figure 6. Effect of BChE/urease ratio on pH equilibrium

We repeated the identical experiment utilizing polyurethane enzyme polymers (squares in Figure 6). Here we varied the BChE/urease ratio from 1:1 to 6:1. Adding the substrate solution to the enzyme polymer started the reaction. In order to measure the pH equilibrium we measured the color of the polymer with the spectrophotometer. These values were correlated by calibrating sensor color to pH with enzyme polymers incubated in buffer solutions of varying pH. Figure 6 demonstrates that the behavior of the soluble and immobilized system is significantly different. Such difference is a result of many factors. Polymerized enzymes are known to have a less pronounced pH optimum, hence the range of attainable equilibrium pH values is broadened with polymerized enzymes.

In addition, the correlation between pH and spectrophotometer readings may be somewhat skewed by the ongoing enzyme reactions. Nevertheless, a pH transition between pH 7 and a BChE/urease ratio of 4:1 seemed to be a perfect choice realizing the Traffic Light Sensor with positive response signal.

In most biocatalytic reactions the enzyme concentration is directly responsible for the reaction rate. Figure 7 demonstrates that the Agentase TL sensor is no exception from the norm. Increasing enzyme concentration clearly improves the reaction rate. The reaction rate can be directly correlated to the color change over time and the response time of the sensor. The optimization of enzyme concentration took into account both effects: the loss of sensitivity and the decreasing of response time in case of increased enzyme content. In conclusion the enzyme concentration of 1.5mg urease and 6mg BChE per gram polymer seems to be a good compromise by combining a good sensitivity with a response time of less than 2 minutes. Polymers with increased enzyme quantities however make sense if a rapid signal of less than 30 seconds is desired in environments with higher nerve agent concentrations.

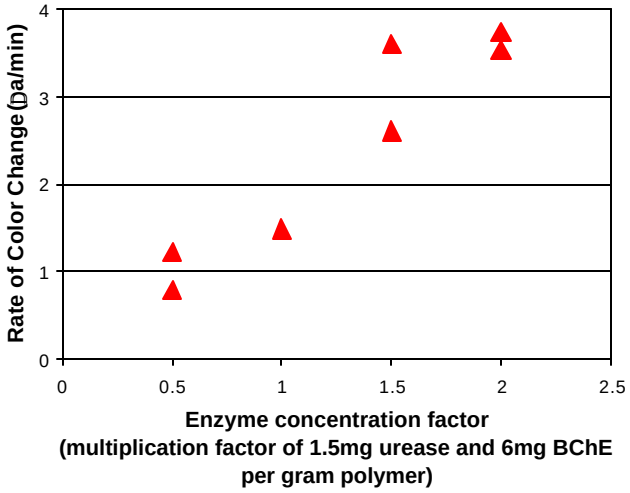

Figure 7. Rate of color change as a function of enzyme concentration

Effect of substrate composition

The substrate composition displays a less pronounced effect on sensor performance than the enzyme. However, it is important to deliver sufficient quantities of substrate to the sensing polymer. Insufficient quantities of either substrate upsets the pH equilibrium resulting in either red controls due to a rise in pH caused by insufficient quantities of butyryl choline, or the absence of red signals in the presence of DFP due to low urease activity caused by the failure to deliver urea. In addition to sufficient quantities, an excess of butyryl choline over urea needs to be considered to improve the signal stability. Premature exhaustion of butyryl choline can potentially lead to a false positive signal once BChE is running low on substrate. As discussed below, the excess of butyryl choline is particular important for the Yellow to Red version of the Agentase nerve agent

sensor where a long signal stability is desired. It is less significant for the Traffic Light Sensor with its green control signal.

The third substrate indoxyl acetate does not play such an important role in the signal development. In the initial stages it has no effect and is solely responsible for the development of the green color, which takes around 20 minutes to appear. The time frame of 20 minutes is chosen to avoid any interference with the red color development during the initial few minutes. Since the green color also results in a darkening of the yellow polymer it could be easily misinterpreted as the development of red color. As indoxyl acetate is hardly soluble in water, only a fraction of the total amount directly affects the liquid phase of the enzymatic polymer where the necessary reactions take place. There is a direct relationship between the rate of green color development and the amount of indoxyl acetate present. Reducing the indoxyl acetate content results in delaying the green color development to ultimately no green color in the absence of indoxyl acetate.

Choosing optimal pH-indicating dye:

The pH indicating dye is another important ingredient of the biocatalytic sensor. The pH transition has to be compatible to the pH equilibrium maintained by the urease and BChE enzymes. Cresol red (for structure Figure 8) with a pH transition from 7.2 (yellow) to 8.8 (reddish-purple) has such a transition considering that the pH equilibrium is around 7 with our present enzyme concentration and ratio. Cresol red with a self-explanatory color transition from yellow to red is ideal for the Agentase sensor. Others dyes tested include cresol purple, rosolic acid, and phenol red. Each of these undergoes a color transition from yellow to red/purple between pH of 7 and 8. Naturally the concentration of dye will affect signal intensity. Therefore we investigated the signal development as a function of cresol red concentration.

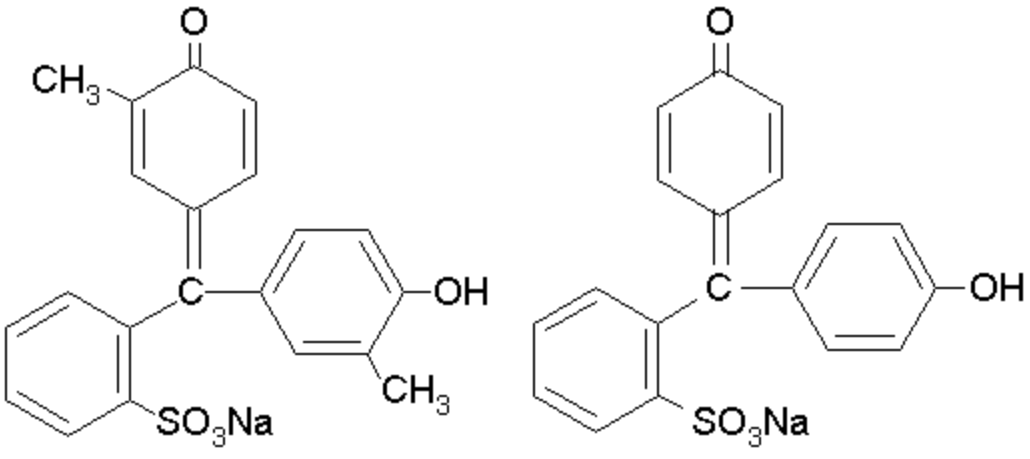

Figure 8. pH indicating dyes (left: cresol red; right: phenol red)

An increase of the dye amount leads to a significant shift towards the red color in both signals, clean and contaminated environment. The baseline shifted around 10 units of "a" towards red, while the reaction itself proved to be not effected as illustrated by the parallel lines. The increase in red signal improves the detection limit, however the increase in dye concentration leads to a more intensive almost orange yellow, which could be interpreted by some users as a positive signal. To eliminate the chance of false positive signals completely we reduced the dye concentration even further resulting in a transition from pale yellow and pale red (compare Figure 5 (early prototype) with Figure 24 (actually produced and delivered to end-users)).

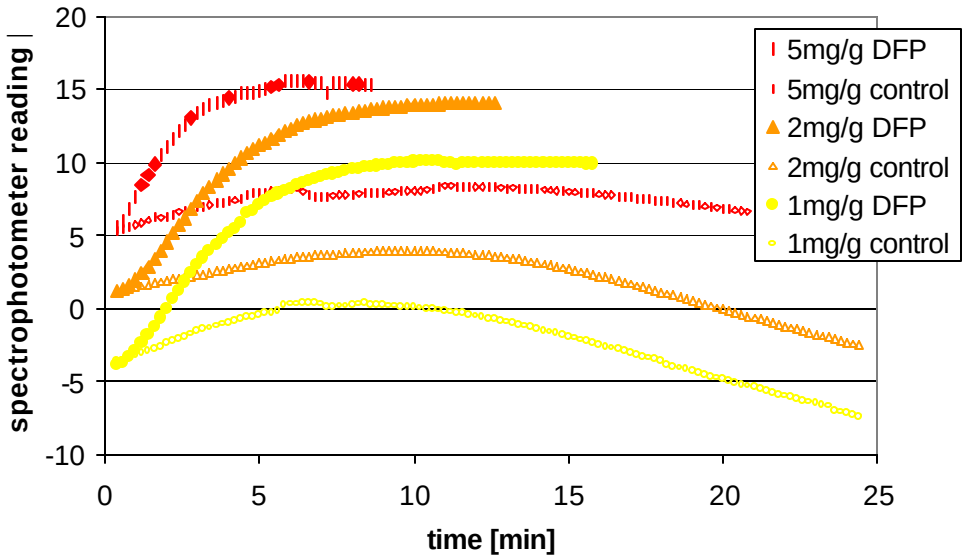

Figure 9. Signal development after sensing 100ng/cm^2 DFP as a function of cresol red concentration (each line represents average of two experiments).

While cresol red demonstrated a superior performance in an initial screen of several pH-indicating dyes, the option of mixing two different dyes was considered. The mixture of phenol red and cresol red offered great potential. Both dyes are very similar in structure (Figure 8), but have two different pH transition with pH 7.2 (yellow) to pH 8.8 (reddish-purple) for cresol red and pH 6.8 (yellow) to pH 8.2 (red) for Phenol red. Phenol red on its own is not suitable for use in the Agentase TL sensor due low pH transition resulting in an orange control reaction. The optimized mixture of both dyes shifted the pH transition closer to the pH equilibrium resulting in a signal improvement.

The final sensor, however, does not contain phenol red due to occasional false positive signal caused by improper activation by end user.

Prototype development

Figure 10 shows a picture of the first generation prototypes which have been tested successfully by the Air Force Operational Test and Evaluation Center at the West Desert Test Facility in Dugway Proving Ground, Utah. The sensor consists of an applicator (1), substrate polymer (2), enzyme polymer (3) and the containment vessel (4). This prototype is made of a Wide-Mouth Jar (15mL) with a plastic vial (4mL) glued into its closure. This sensor needs to be activated with clean water prior to sensing.

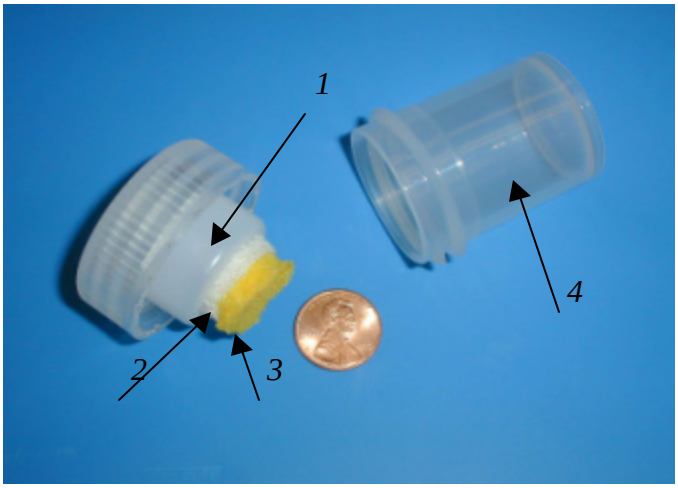

Figure 10. Picture of Agentase Traffic Light Sensor (1st generation prototype)

While the first generation prototype received the highest achievable score, demonstrating utility and being recommended for immediate deployment, the following improvements have been suggested by the end-user:

- *Lengthen the Traffic Light Sensor's cap to prevent cross-contamination*
- *Add a tab to the Traffic Light Sensor's cap to facilitate opening the unit*
- *Improve the adhesive bond between the applicator (cap) and the sponge (sensor)*
- *Provide an individual water source for each Traffic Light Sensor or a spray-on application to avoid cross-contamination*

Additional requirements are:

- *Utilize as many off-the-shelf parts as possible to keep cost down*
- *Keep utilizing screw-closing mechanism to isolate sensor from environment*

All of these requirements could be successfully accommodated within the 2nd generation prototype. This prototype includes a *water reservoir* with *release valve* secured by a *rubber washer*. The valve releases water once the red tip gets pushed onto a surface. The version shown in Figure 11 contains in addition to previous versions a *plastic washer* between *enzyme* and *substrate polymer*. This washer has several

functions. First, it improves the shelf-life significantly. As preliminary accelerated stability studies at elevated temperatures have shown the sensor exhibits a significantly prolonged shelf-life once the two polymers are detached rather than glued together. Secondly, the washer facilitates the manufacturing process. It secures the substrate polymer, which is simply attached to the red valve tip. Also it contains ridges, which secure the enzyme polymer once melted, achieving a very strong bond between the sensing polymer and applicator. The melting process firmly attaches the enzyme polymer to the plastic washer exceeding the bonding strength of previously glued versions.

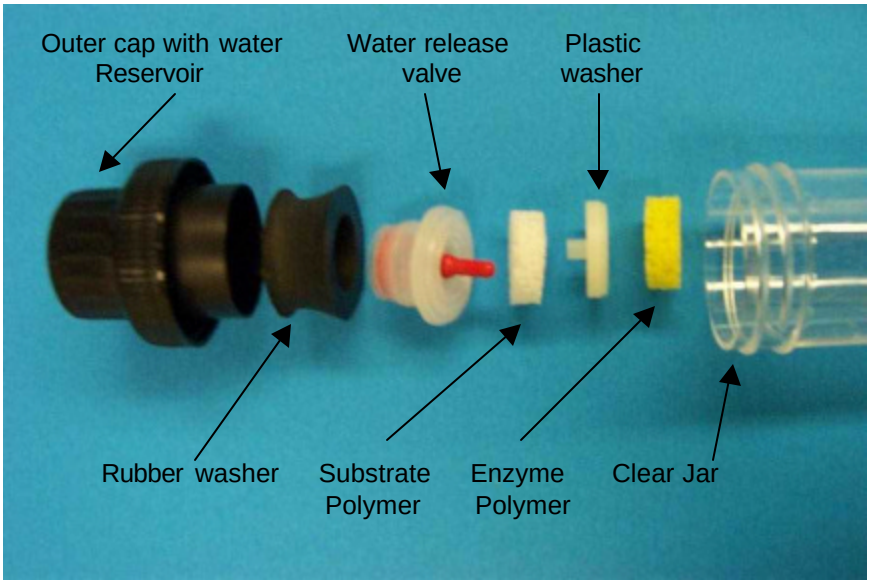

Figure 11. Explosion picture of Agentase Traffic Light Sensor (2nd generation prototype)

By realizing the integrated water source, a new closure design and achieving an improved bond between polymer and applicator all end-user recommendations could be implemented. This sensor fulfills the specifications mentioned in the recommendations by the Dugway test team. The shape of the closure is easy to open while it prevents cross contamination. Figure 12 shows the assembled sensor and a smaller light-weight version of it, with 14gram instead of 25gram weight.

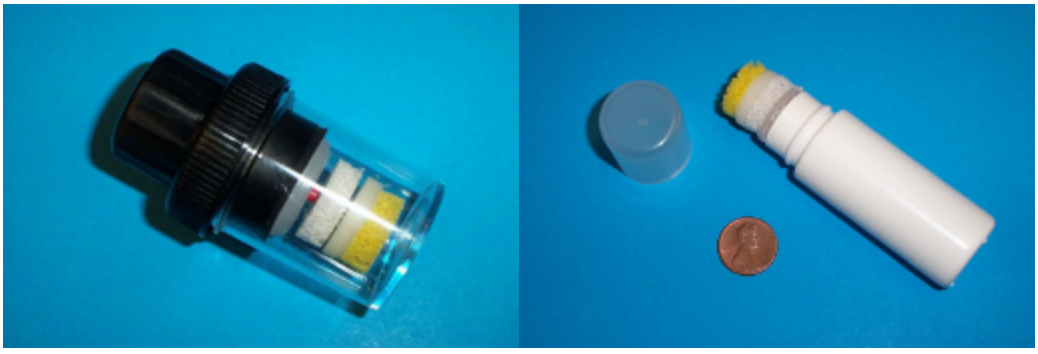

Figure 12. Picture of 2nd generation Traffic Light Sensor (regular and small size)

The Traffic Light Sensor has been developed further based on initial experiences with prototypes. The utilization of rubber washers to adapt the valve assembly to the water reservoir showed some faults especially during shipment by air. Pressure within the reservoir (high temperature) or vacuum in the outside environment (airplane) has the potential to cause leakage. For example around 10% of casings failed during a shipment by air. The assembly process using rubber washers is also very difficult in terms of delivering consistent quality. To achieve high reproducibility for the assembled product, custom molded plastic parts are far superior to rubber products. Figure 13 shows the realized design with two pieces, an inner container and a plastic ring to secure the valve, which has shown to fulfill design requirements in initial tests. The sensors showed no leakage during storage in a lab shaker at 400rpm at a temperature 50^{o}C. During assembly the inner container has to be filled with 2mL water. The valve/polymer assembly has to be pressed in to the container resulting in a tight fit that guarantees a good seal. To prevent the valve from moving due to excess pressure it needs to be secured by a 90-degree turn of the nylon ring (See below).

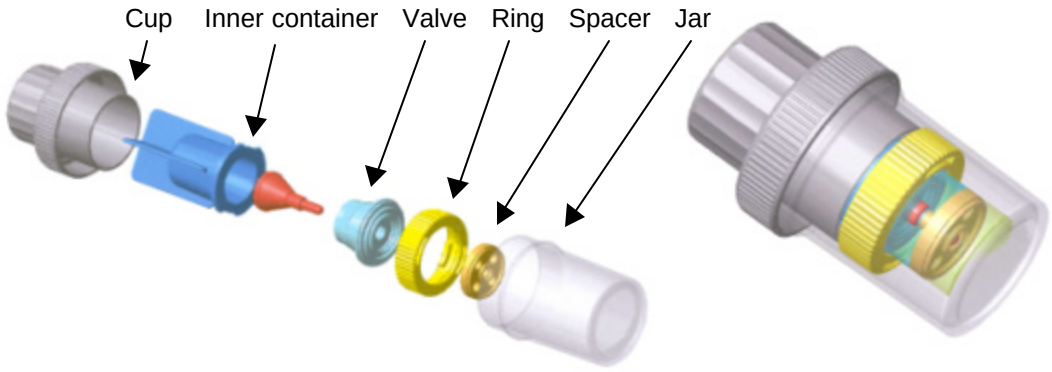

Figure 13. Current improved casing design using custom molded parts

We introduced an aluminum cap to improve the sensor stability (Figure 14.). Exercises at the Allegheny General Hospital in Pittsburgh showed that this aluminum cap is impractical in the field as potential end users in protective suits encountered problems in removing this cap prior to activation of the sensor (see Quarterly Report VI). At this exercise, medical personnel utilized the Agentase Training sensors simulating a nerve agent attack on the Pittsburgh underground train system. Under stressful situations, it proved to be difficult to remove the aluminum cap as users occasionally pulled the nylon disk with connected enzyme polymer from the valve rather than the cap from the nylon disk.

The sensor design shown in Figure 14 and Figure 15 addresses this end user critique by separating the substrate polymer from the sensor itself. Only the enzyme polymer is attached to the device instead of the two used in the previous design. This helps with signal readability, unit stability and the ease of activation. The substrate polymer is glued, using a hotmelt glue gun, to a yellow cap, which also acts as an activating dish. Using an activation dish has the additional advantage in ensuring

reproducible activation processes by the end-users, as the surface conditions are always identical.

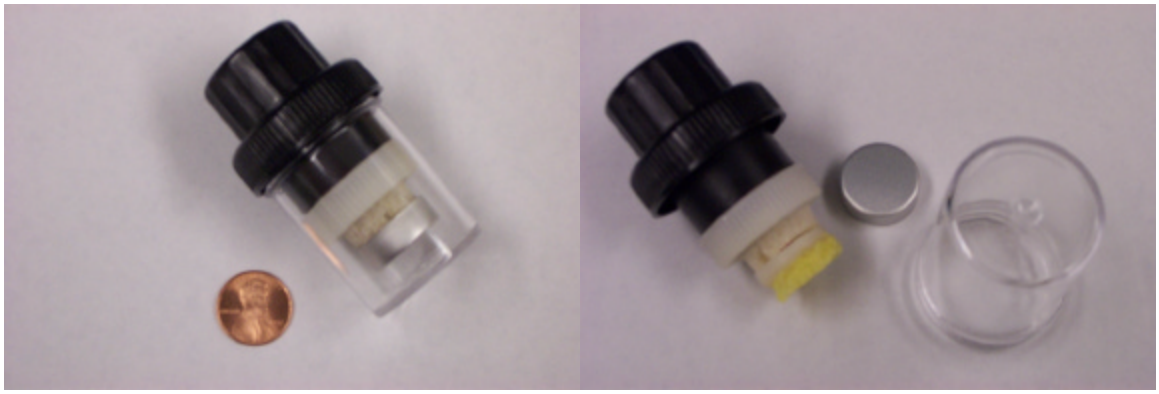

Figure 14. Agentase TLS with aluminum cap attached (left) and removed (right)

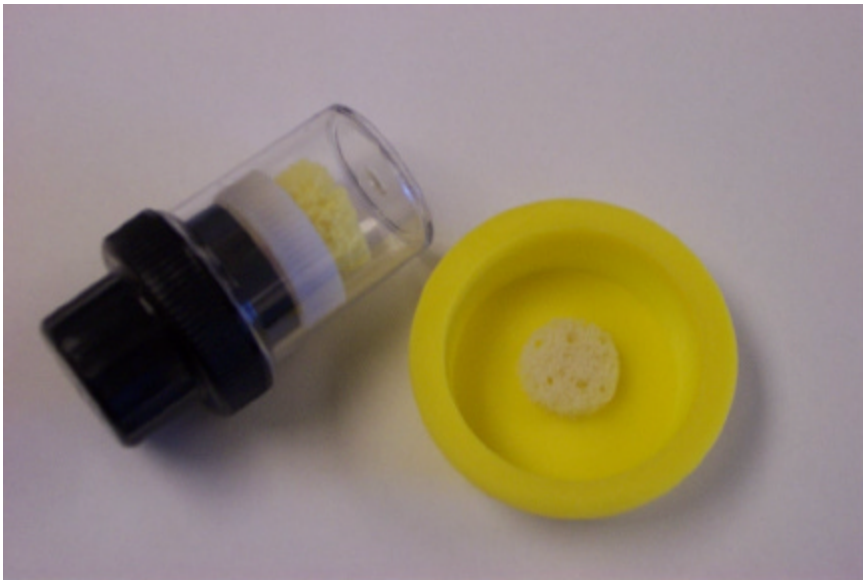

Figure 15. Picture of 3rd generation and currently fielded Traffic Light Sensor

Secondary products

Red-Yellow Sensor

A potential marketing partner expressed the wish for removal of the green signal because of concern that end-users could wrongly interpret the green signal response of the Agentase TLS as “an all clear signal”, whereas the green color only indicates that the sensor worked properly and that there are no nerve agents present. They also expressed concern about the stability of the colorimetric signal around the detection limit (stable for about 30 minutes, than turns green). To remove the green signal both polymers, the substrate polymer mounted in the activating dish and the yellow enzyme polymer needed to be reformulated. Besides the signal development the sensor operation has not been changed.

The redevelopment of the substrate polymer required some work since the sensor did not function properly after removal of indoxyl acetate without changing the urea and butyryl choline (BCh) concentration. Eliminating indoxyl acetate results in a faster BCh consumption as there is only one substrate available to the BChE enzyme. While a higher BCh concentration resolves this problem, we reduced the urea concentration to achieve an excess of BCh. In addition, the new substrate composition ensures that urea is the first substrate to be exhausted resulting in an drop of pH (yellow) rather than rise in pH (red) once the equilibrium reaction ceases to work. The removal of indoxyl acetate required a small change within the enzyme polymer composition. As explained above the removal of indoxyl acetate results in a fast butyrylcholine degradation, hence a faster acid production which lowers the pH equilibrium of both enzymatic reactions. Lowering the pH equilibrium results in an decrease of response time and signal intensity at sensing applications close to detection limit. Therefore we increased the urease amount by about 10% to compensate the higher BChE activity.

Figure 16 clearly shows that the signal around detection limit is stable for several hours. The stability improves even further at higher contamination. Agentase is using a yellow “activating dish” that can be used when signal interpretation is in question.

Figure 16. Colorimetric response of Yellow to Red Sensor version (for MSA)

Training sensor

Agentase believes that "hands-on training" is an important tool for training prospective end users. Because of this we have developed a training version to simulate the usage of the Agentase Traffic Light Sensor (TLS) for detection of nerve agents. The Training Sensors provide an identical response under identical application procedure using urea as simulant. They are be a helpful training tool along with watching the instructional video for the Agentase Traffic Light sensor. Prior to using the training sensor, the "contaminated" surface needs to be prepared by applying the liquid agent simulant. This liquid agent simulant is enclosed within the training package and contains urea, an environmentally benign chemical. In a response pattern identical to that of the nerve agent Traffic Light Sensor, the training sensor exposed to the contaminated surface will rapidly transition from yellow to red (within 2 minutes). When exposed to a clean surface will gradually become lime-green (within 20 minutes). Significant delays in green color development indicate an insufficient activation procedure. To improve the response, the sensor requires additional activation. The only significant difference between the training and nerve agent Traffic Light sensors, is that the red color of a "contaminated" training sensor is relatively unstable (gradually turns purple) whereas the traffic light sensor maintains the contaminated red color for hours. The training version is also not suitable for detection of nerve agents.

The Training Sensor is suitable for classroom training indoors and outdoors. In addition it will be suitable for decontamination exercises. In this case the objects have to be prepared using the liquid agent simulant. The sensor will indicate an insufficient decontamination by turning red if less than 99% of simulant is removed. It will indicate a clean surface with decontamination efficiency greater than 99% by turning gradually to green. Also it should indicate the presence of urea at a range from 20mM to 2M (Figure 5). In utilizing 2M urea as simulant at decontamination exercises, the detection of 20mM urea indicates if cleaning efforts were successful up to a 99% level.

Figure 17. Performance of Agentase Training sensor

Water test kit

Agentase has devised a simple system for detecting low concentrations of target nerve agents in solution. The existing prototype was constructed using off-the shelf components. Agentase is planning to use a molded funnel constructed to improve product usability once a market for the test kit is foreseeable (Figure 18). This design concept includes an off-the-shelf powder funnel and a wide mouth jar. Only the polymer holder needs to be custom-designed and produced. Besides holding the enzyme polymer in place, this part will also act as an activation dish eliminating the need for the user to remove the sensing polymer.

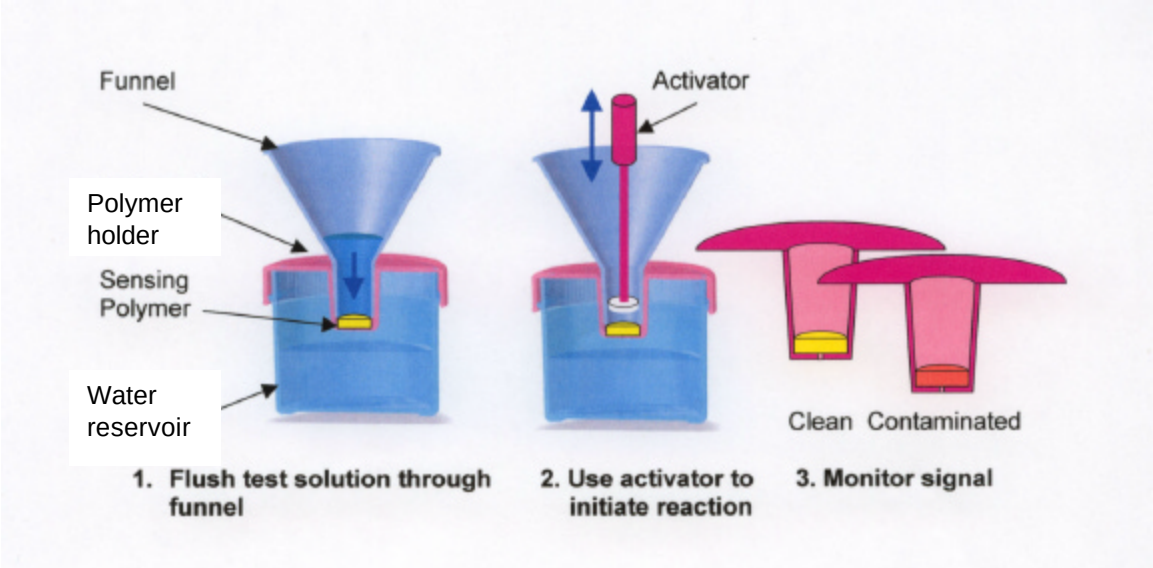

Figure 18. Design concept of Agentase water test kit for detection of nerve agents in solution

The protocol in using the water test kit consists of three simple steps similar to the Agentase TLS:

- Add test solution to funnel (roughly 50mL) and allow to drain through the unit (about 5 minutes)
- Initiate the sensing reaction by repeatedly pressing activator onto the enzyme polymer sensor disk for at least 10 seconds (similar to standard TLS system)
- Monitor color development

Depending on the pH of the test solution the sensors may be slight orange in color after before activation, but they will turn yellow upon activation. Contamination is detected when sensors turn from yellow to red-orange within 2 minutes of activation. Higher levels of contamination result in faster and more intense color development. In the absence of contamination, activated sensors stay yellow and do not turn green. The absence of the green signal represents the major difference to the Agentase TLS. Figure 19 illustrates the protocol of the existing prototype water test device simulating the design concept very closely.

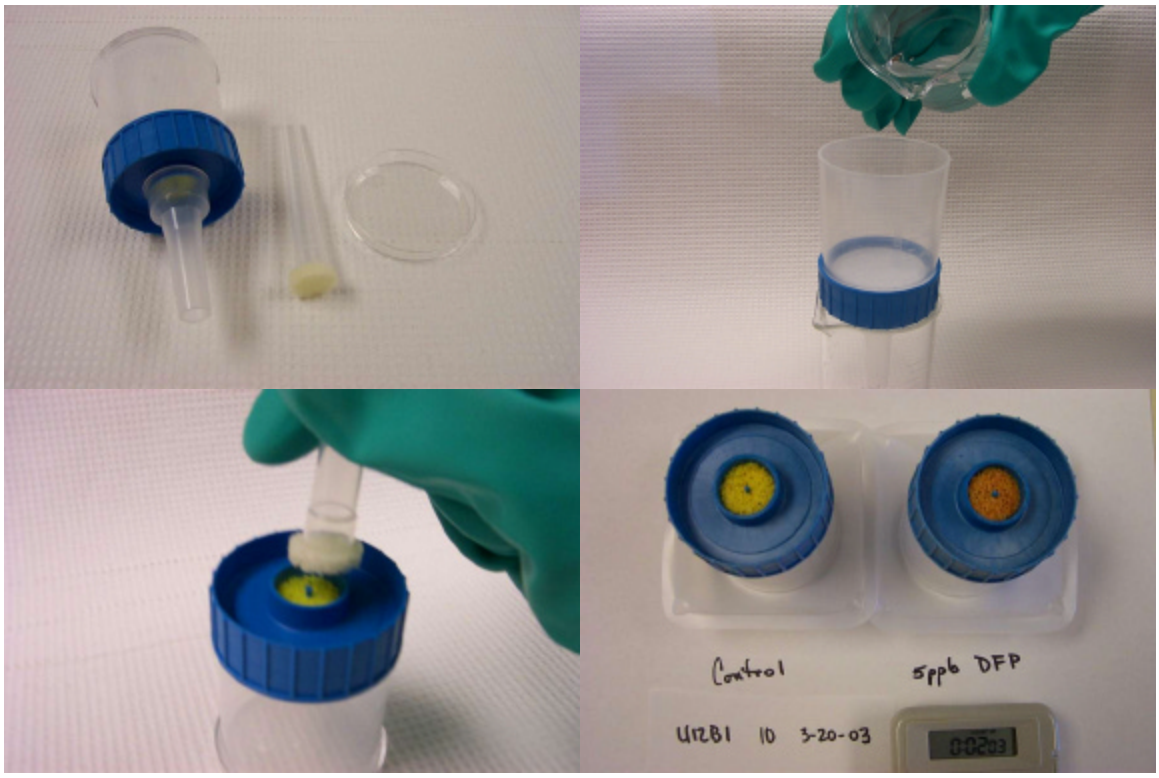

Figure 19. The use of the Agentase water test kit (Prototype)

Figure 20 demonstrates that the detection limit using di-isopropyl fluorophosphate (DFP) is well below 10ppb.

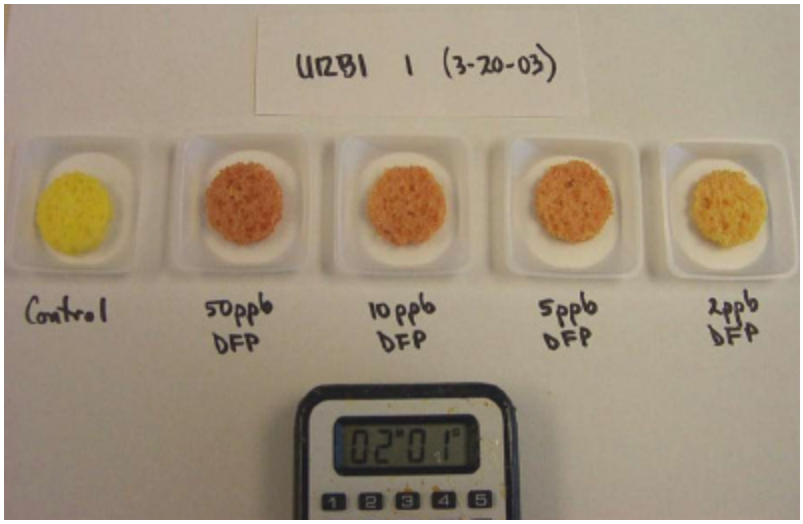

Figure 20. Detection limit of Agentase water test kit

Air sensing

Figure 21 shows our first attempt in demonstrating the continuous monitoring capability of Agentase sensors in air. The first sensor prototype consisted of an outer substrate polymer and an inner enzyme polymer similar to the Agentase TLS concept. The only difference being the absence of indoxyl acetate, the substrate responsible for the green color development. For obvious reasons the green signal in absence of nerve agents is undesirable for a continuous operating sensor. The sensor placed on top of the open vial containing DFP clearly displays a positive signal in form of a brownish red color, while the two control polymers maintained their yellow color. The two timers in Figure 21 indicate the time lapsed after activation of the sensors (lower timer) and the time lapsed upon releasing DFP vapor by opening the vial (upper timer). The experiment took place in a fume hood with a high airflow. Therefore, we had to add 1mL of H_2O to compensate the loss of water due to evaporation. If the sensor is allowed to dry out, the sensing performance decreases rapidly.

Figure 21. Continuous sensing of DFP in air 45min after activation.

We continued working on continuous sensors during work carried out with TIAX (formerly Arthur D Little). The aim of this collaboration was the development of a wearable badge for nerve agent detection with an operational life-time of 12 hours. Figure 22 illustrates that we were successful in achieving the objectives in detecting 10ppb DFP vapor using a detection system, which was operational for 12 hours. Unlike the first prototype, this system contains no substrate polymer. A substrate solution is added at a flowrate of 1mL/h using a micropump.

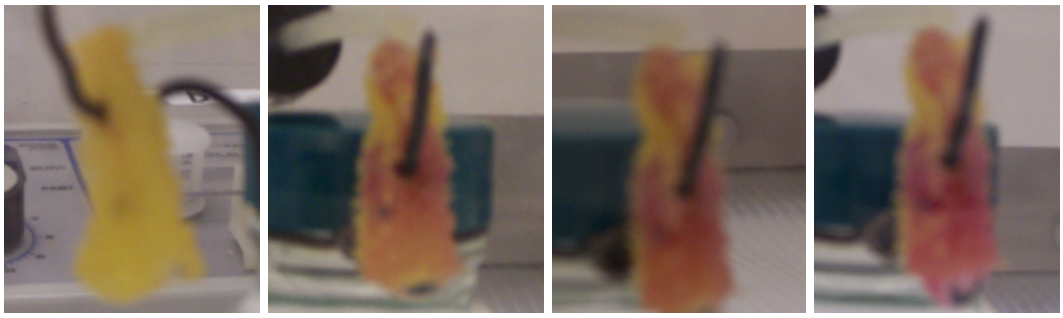

Figure 22. Response when 10ppb DFP vapor is added to gas chamber after the base has been operating for 12 hours. (from left to right: t=0, t= 3min., t=5min., and t=10min.)

Electronic signal reader

Figure 23 displays the result of 3rd party feedback received by Agentase. Potential end-users raised concerns about limitations in utilizing the Agentase TLS during night or in dark environments. Agentase therefore designed a simple battery-operated reader for the TL Sensor containing its own light source and light-sensitive diodes. In addition, an electronic signal reader removes the subjectivity due to reading of a color change by humans and opens the potential for remote sensing applications. The electronic signal reader also allows a quantification of contamination present since the rate of color change can be determined. For example a contamination at detection limit yields a change from yellow to orange within 2 minutes, while a highly contaminated test environment yields a strong red response within 30 seconds.

Figure 23. First prototype of electronic signal reader

The basics of the sensor works in the following manner. First, in order to detect the color change, a light sensitive voltage converter is used. This has a primary light source and a color filtered photodiode that converts the filtered light intensity to a voltage output. The sensor will monitor the amount of reflected light to the object‘s color. The sensor then gives a voltage output proportional to the light reflected. This voltage is monitored by a operational amplifier that has an accompanying voltage divider network.

Basically, when the voltage of the sensor goes above or below the voltage created by the voltage divider it will give power to a light, signaling that the color has changed. The electronic signal reader will monitor when the color of the polymer has reached a target red or target green, and will light a light according to the change. If the voltage is within the desired limit's a constant yellow light will signify that the polymer is still yellow. Also an audible alarm is implemented, when the target red has been detected it will emit a tone.

Product performance

Detection limit

In general the current TLS achieves virtually the same performance as the previously reported versions (Figure 5). The only difference can be observed in the intensity of the colors. In order to eliminate false positive results we reduced the dye content of the enzyme polymer by half resulting in a more pale yellow color for the control reactions. The reduction in dye concentration significantly improved the contrast between control and sensing applications around detection limit, even though there is a slight decrease in color intensity for the red signal at contaminated samples. The detection limit remains the same as can be seen in Figure 24 and Figure 25. Contamination around detection limit results in a peachy kind of orange color. Figure 24 shows the results using the standard surface testing method, which means activation of the sensor prior to wiping the surface in question. As the picture shows the detection limit is clearly below 1µg DFP per surface.

Figure 24. Performance of TLS at surface sensing (DFP loaded on 70cm^2 surface)

Figure 25 displays the results after testing various solutions contaminated with DFP. The left picture shows the results after utilizing the TLS with the standard application procedure for testing of solutions. Similar to the surface method the sensor was activated prior to briefly dipping it into the solution in question. The detection limit in solution is below 500ppb. Both methods for surface and solution are in principal identical consisting of three different steps:

- Activation
- Testing
- Signal monitoring

Following these steps guarantees rapid results in seconds after sampling high concentrations of chemical warfare agents in an easy to learn and intuitive method.

Figure 25. Performance of TLS at testing solutions

The simple sensing procedure makes the TLS an ideal tool for the military and civilian homeland defense market where potential end-users are unlikely to receive intensive training on a regular basis. Extended periods of more than a year between training and actual use in the field are feasible.

Interference studies

Agentase conducted its interference tests by placing a given mass of interferant within tap water using the same approach as we used in testing the TL sensor. The Agentase approach results in significantly greater interferant concentrations and exposure in comparison to tests performed by the U.S. Army Soldier and Biological Chemical Command (SBCCOM) *(10-11)*, where commercially available sensors for chemical warfare agents were tested against "1% concentrations" of saturated air solutions. Interference occurs when the sensor does not function exactly as it should, i.e. the sensor gives a false positive signal or the effect of DFP (a nerve agent simulant) on the sensor is masked. After activation, the sensor was briefly dipped into solutions of each concentrated interferent listed below. Table 1 summarizes the results.

Urea is not listed as typical interferent of interest, however due the mechanics of the Agentase Traffic Light Sensor it could be a potential interferent. We tested samples concentration up to 2M and observed no loss in performance. This amount of urea is much greater than that found in urine and saliva. These results clearly show that the sensor is highly resistant to chemical interference. Exposure to excessive amounts of strong acids or bases during sampling render the sensor ineffective. The sensor will immediately change color (to purple or pink).

Table 1. Concentration of interferant compatible with sensor

Interferent	Percentage
Antifreeze	>1
Hypochlorite bleach solution	0.01
Ethanol	>1
Fire-fighting foam	>1
Floor wax	>1
Gasoline	>1
Concentrated hydrochloric acid	0.01
Insect repellent	>1
Jet fuel	>1
Motor oil	>1
Off-road diesel fuel	>1
Sunscreen	>1
Toluene	>1
Vinegar	>1
Windex	>1

In order to detect agent on frozen surfaces, we stored the Agentase sensor in the refrigerator for at least 2 hours prior to testing to achieve a realistic sensor temperature. A sensor and a ceramic tile (surface loaded with 10ug DFP) were place outside at –9^{o}C for 10 minutes prior to activation until both DFP solution and water within the sensor were partially frozen. As Figure 26 demonstrates the sensor performed well at sub zero temperatures. However the response time increases by at least 50%. This represents no problem at DFP concentration 10 times the detection limit (shown here), but may delay the signal at detection limit beyond 2 minutes.

Figure 26. Detection of DFP on frozen surface at –9^{o}C (left picture 2min, right picture 3min)

To verify the fact that Agentase sensors can be stored frozen, we kept sensors at –20^{o}C for 48 hours until testing. Prior to testing we thawed them at room temperature for about 15 minutes until the liquid inside the sensors was only partially frozen. Sensors

were tested at room temperature and at -9°C and performed identical to sensors, which were stored at room temperature. It is important to note that water is essential to proper activation of the sensors, therefore the sensors **can not be used** in the frozen state. If sensors are partially frozen or remain just above the freezing point they perform normal. Storage at cold or subzero temperatures shows no detrimental effects. Experience with any enzyme products even suggests that these kind of storage are likely to increase the shelf-life significantly.

The Agentase TLS has been tested on a variety of surfaces outside the laboratory. The sensors performed well on all surfaces such as metal (trunk of a car), finished wood, concrete, stone and asphalt. Excessive dirt or any material that significantly masks clear viewing of the sensor polymer may invalidate sensor performance. Also surface sampling of highly porous materials with chemical agent deeply entrained within the material may not yield an accurate test.

Shelf-life

Stability testing was conducted on TLS, Yellow/Red, and Training sensors. The testing consisted of storing sensors at room temperature, 40°C, 50°C, 60°C and 70°C. The sensors were then activated and tested for color development after a period of days. When a sensor no longer developed red color within 2 minutes or the detection limit increased it was failed. Table 2 shows the results of this stability testing completed thus far. The TLS has been stable for more than one year when stored at room temperature. Data of various other enzymatic polymers suggest that the shelf-life is very likely to exceed 2 years. Table 2 also shows that the sensor can be stored at high temperatures for brief periods of time e.g. while used in the field. High temperatures however are detrimental to the long-term shelf-life and should be avoided during extended storage.

Table 2. Stability studies of Agentase TLS, Yellow/Red and Training sensors

Stability: TLS	Room Temperature	40	50	60	70
Days until sensor fails	Ongoing (>365)	56	21	3	1

Stability: Yellow/Red	Room Temperature	40	50	60	70
Days until sensor fails	Ongoing	Ongoing	Ongoing	31	5

Stability: Training	Room Temperature	40	50	60	70
Days until sensor fails	Ongoing	Ongoing	Ongoing	1	1

Figure 27 below is a sample of the stability testing done with TLS. These sensors were stored at room temperature for 361 days. The sensors detected 5ug DFP within 2 minutes, and green color developed within 20 minutes. It has to be noted, that the packaging of the final sensors has been improved significantly in comparison to those

pictured. For example the substrate and enzyme polymer are glued directly together a fact which is proven to be detrimental to shelf-life as shown during studies at elevated temperatures.

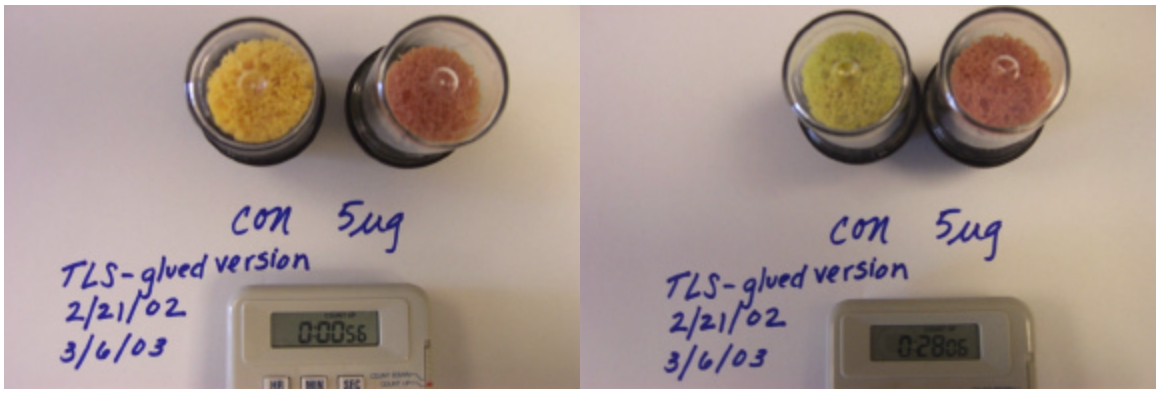

Figure 27. The use of the Agentase TLS after one year of storage

The difference between TLS and the Yellow-Red sensor version can be explained by the fact that the development of green color is far more sensitive towards shifting in pH equilibrium as the red color development. Activity studies have shown that the urease enzyme loses its activity slightly faster than BChE, hence the decrease in pH equilibrium. A slight decrease in pH lowers the BchE activity significantly delaying or preventing the development of green color. The red signal on the, other hand, is far less sensitive to a shift in pH.

Ellman's assay was used to test BChE levels of Yellow/Red polymers stored at room temperature opposed to Yellow/Red polymers stored in a 70°C oven. The longer the polymers were stored in the oven the lower the BChE levels became. The results are shown in Figure 28. Each line represents an average of duplicates tested.

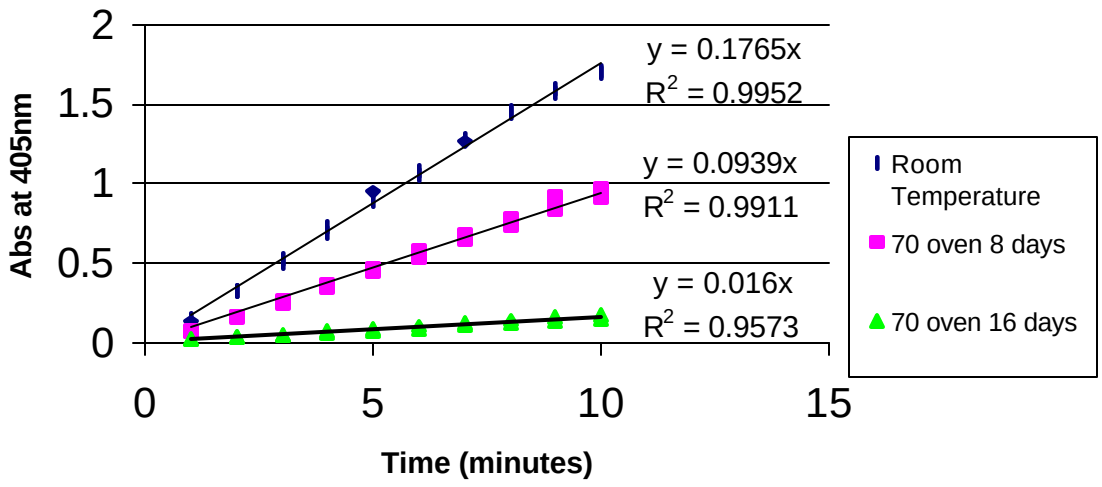

Figure 28. BChE activity of sensors stored at 70°C

End user feed back and testing

Field testing of the sensor

West Dessert Test Facility, U.S. Army Dugway Proving Ground (DPG), Utah, on 23-24 May 2001

Prototypes of the Agentase Traffic Light Sensor (TLS) for detection of nerve agents were assessed by Detachment 1 of the Air Force Operational Test and Evaluation Center (Det 1 AFOTEC) as part of the Weapons of Mass destruction (WMD) Enhanced Collection Support Capability MUA (WMD 1st Response) for the Defense Advanced Research Projects Agency (DARPA). The assessment took place at the West Dessert Test Facility, U.S. Army Dugway Proving Ground (DPG), Utah, on 23-24 May 2001 subsequent to a training session at the Defense Intelligence Agency (DIA) Headquarters at Bolling Air Force Base, Washington D.C on 13 April, 2001.

The prime objective of this assessment included judging the suitability of the Agentase TLS as an intelligence collection tool. This exercise utilized the first training version of the Agentase TLS, which yields a positive response under absence of nerve agents and the presence of harmless simulants. The design and application procedure of both sensor versions was identical. The DIA team utilized the Agentase Traffic Light Sensor within their normal procedures as part of a chemical agent scenario.

RECOMMENDATIONS AND CONCLUSIONS

The purpose of this assessment was to determine if the prototype technologies provide an enhancement to the DIA team's intelligence collection mission. The Traffic Light Sensor provided a quick and simple detection tool, which provided accurate results in the field. These sensors were prepared prior to the demonstration for sampling of the known contaminants in the area; therefore, the scenario did not provide a realistic CBW detection mission. It is recommended that the sensors be evaluated as they would be deployed (that is, more than one pretreated sensor to select from when the contaminant is not known). Users of the technology provided the following recommendations to improve the Traffic Light Sensor.

- *Lengthen the Traffic Light Sensor's cap to prevent cross-contamination*
- *Add a tab to the Traffic Light Sensor's cap to facilitate opening the unit*
- *Improve the adhesive bond between the applicator (cap) and the sponge (sensor)*
- *Provide an individual water source for each Traffic Light Sensor or a spray-on application to avoid cross-contamination*

Overall, the users of the Traffic Light Sensor were pleased with the technology and would take the Traffic Light Sensor with them if they had to deploy for a mission tomorrow.(4)

The conclusions demonstrate clearly the success in convincing prospective end-user of the utility in using the Agentase TLS as a quick and simple detection tool for nerve agents in the field. The recommendations highlight the importance of early field trials. The implementation of the four recommendations into the second-generation prototype design lead to major improvements of the overall sensor. The new generation TLS is more rugged and even simpler to use due to the internal water supply.

City of Pittsburgh Operational Exercise Oct 10th, 2002

The City Of Pittsburgh conducted a WMD exercise on Oct 10, in which simulated chemical attacks were made on the city's subway system and 2 surrounding shopping malls. The main purpose of the drill was to assess the readiness levels of local hospitals in responding to an event of chemical terrorism. Allegheny County's chemical field exercise took place at all hospitals in the region, providing healthcare professionals with a real time experience in the triage of patients suspected as being the victims of a nerve agent assault.

Agentase directly participated in the exercise at Allegheny General Hospital (AGH). The training version of the sensor, which uses urea as a benign simulant was employed during the exercise. AGH staff found the sensor particularly useful as a tool during triage, pre-decon line assessment and post-decon line assessment of contamination. The use of urea

during the exercise as a simulant worked extremely well and contaminated individuals were effectively identified by AGH staff.

Subsequent meeting with hospital staff provided Agentase with two substantive suggestions on sensor performance:

- The sensor could be improved in decon line applications with the advent of a technique to directly link an individual to the particular sensor used. A clasp to attach the device to an individual's ID tag post-decon or a string to hang it from a patients neck would be highly advantageous in such applications.

- Removal of the metal cover from the enzyme disk prior to activation is not straightforward when wearing thick protective gloves during a time of high anxiety. Suggestions were posed for a tab that could be used to simplify the process.

Agentase is presently considering these options for incorporation into the final device.

> *Based upon the product evaluation, Thomas Stein, M.D., director of AGH's Emergency Medical Support Services and Life Flight Operations and a colonel in the U.S. Army Medical Corps said, "In the event of a bioterrorism incident involving the release of chemical nerve agents, our ability to expeditiously evaluate patients for nerve agent exposure will be vital to successfully managing the large volume of patients such an event would likely impose on healthcare facilities such as AGH. The Traffic Light Sensor is an ideal tool in this regard, providing us with a reliable, compact and relatively inexpensive means of quickly assessing someone's exposure to these potentially deadly nerve agents."*

Collect feedback from end-user assessments

Most feedback collected thus far has been extremely positive. Prospective end users from the military, intelligence, and domestic preparedness communities are highly interested in using the sensor as soon as possible.

> *"We presented them* [Agentase Traffic Light Sensors] *to the NAVCENT Emergency Response Team (which has members from all services on it) as well as our theater medical surveillance team plus a few others who deal with chem/bio issues. All were very interested and we're going to add the 8 or so units that Charlie gave us to the toolkit for use and evaluation."*
>
> *Lee Mastroianni, COMUSNAVCENT Science Advisor*

A representative presented the "traffic light sensor" to prospective end users out of the domestic preparedness community at the FDIC (Fire Department Instructors Conference) in Indianapolis April 2002. The 86 respondents were highly interested and liked primarily the simplicity in using the sensor, the quick detection time and the compact/ easy storage. They would like to use the sensor for surface and air detection. The wide majority of end-users are highly satisfied with sensor attributes such as size and weight, response time and ease of use. The majority also considers the attributes of the Agentase Sensor as unique and indicates that there are no similar products on the market. In addition, 75 out 86 respondents would expect their department to purchase the Agentase Traffic Light Sensor.

After reading a short description the respondents were asked, how interested they would be in using the Agentase Traffic Light Sensor:

> *"Here is a sample of a one-time use (disposable) method of detecting nerve agents. It is very specific and only responds to chemical nerve agents like tabun, sarin, soman, etc. It changes from the yellow color you see now to a red color in the presence of nerve agents in a few minutes or less. If no nerve agent is present, it will stay yellow and then turn green in two minutes. This product would have a shelf-life of two years."*
>
> ***"If it could be used to detect Immediately Dangerous to Life and Heath (i.e., ppb) concentrations of liquids on surfaces by wiping an area, how interested would you be in this product?"***

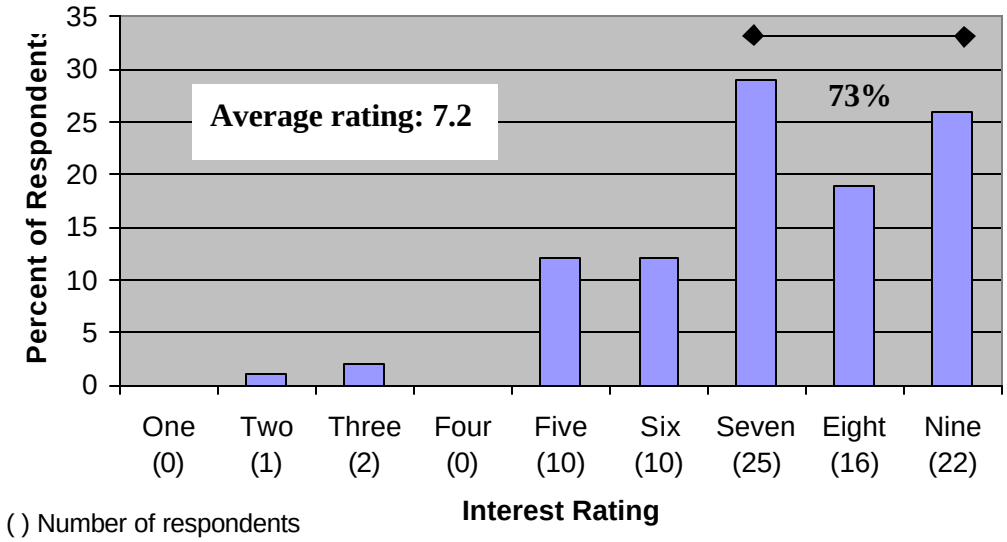

Figure 29. Market survey

In addition we have sent about 900 sensors to various potential end-users within the military and the homeland defense communities for product testing and demonstration:

- Mike Allswede, UPMC Pittsburgh, PA
  for product demonstration to President Bush
- Mine Safety Appliances (MSA), Pittsburgh, PA
  for product demonstration to potential end-users in the domestic preparedness community
- Carlie Kiers, DARPA
  to send samples to Bahrain, Japan and Afghanistan for operational review by end-users in the military community
- Alan Russell, UPMC Pittsburgh, PA
  to demonstrate sensors at Homeland security meeting at the White house
- Mike Reiner, Safety Solutions, Boyton Beach, FL
  for product information
- LTC Luckey, USAMRICD, Aberdeen Proving Ground, MD
  for product information
- Andy Mitchell, Deputy Director office of Justice programs, Department of Justice
  for product information
- MAJ John Buethe, 9th CST, Los Alamitos, CA
  for product information
- LTC Xavier Stewart, 3rd WMD CSD, Annville, PA
  for product demonstration
- Carter Hall, Fisher Scientific, Coope City, FL
  for product demonstration
- Shelley Lowe, DSTL Porton Down, Salisbury, United Kingdom
  for product testing using live nerve agents
- Stephen Lee, ARO, Department of Defense Day on the Hill, Washington, DC
  for product demonstration to the United States Congress
- Earl Freilino, Director PA Homeland Security Office, Harrisburg, PA
  for product demonstration
- Adam Becker, Marine Corps System Command, Quantico, VA
  for product demonstration
- Donald Buley, US Army Material Command, Falls Church, VA
  for product demonstration
- Dean Lyon, Sigma-Aldrich Corp, St. Louis, MO
  for product demonstration

Live agent testing

While Agentase conducted sensor design and use protocol optimization using simulants in our laboratory, numerous studies have been conducted to demonstrate that simulant results correlate favorably with those using live agents. Agentase has taken part in live agent studies in the US, Germany, France, and the UK, including work at Dugway Proving Ground and Edgewood. Those evaluations done outside the US were conducted as part of Agentase's participation in NATO Project Group 31, an effort to evaluate enzyme-related technologies for CW decontamination and detection. Table 3 contains a summary of final experimental findings from the U.S. Army Soldier and Biological Chemical Command; Edgewood Chemical Biological Forensic Analytical Center's sensitivity assessment for the Traffic Light Sensor.

Summary of live agent test data from Battelle Edgewood with proper sensor activation procedures.

Table 3. Live agent data from Battelle Edgewood

Agent	*Total Mass*	*Mass / cm^2*	*Color (2 min)*	*Color (25 min)*	*Green (Y/N)*	*Result*
GD	100 ug	1.25 µg/cm^2	Red	Red	N	
	20 ug	0.25 µg/cm^2	Red	Red	N	
	8.0 ug	0.10 µg/cm^2	Red	Red	N	
	4.0 ug	0.05 µg/cm^2	Some Red	Red	N	
	1.0 ug	0.0125 µg/cm^2	Some Red	Red	N	**Limit**
	0.2 ug	0.0025 µg/cm^2	Yellow	Yellow	N	
VX	100 ug	1.25 µg/cm^2	Red	Red	N	
	20 ug	0.25 µg/cm^2	Red	Red	N	
	8.0 ug	0.10 µg/cm^2	Some Red	Red	N	
	4.0 ug	0.05 µg/cm^2	Slight Red	Red	N	**Limit**
	1.0 ug	0.0125 µg/cm^2	Yellow	Yellow	N	
	0.2 ug	0.0025 µg/cm^2	Yellow	Yellow	N	
Water	Blank Plate a	0.00 µg/cm^2	Yellow	Lime green	Y	
	Blank Plate b	0.00 µg/cm^2	Yellow	Lime green	Y	
	Blank Activated Only	0.00 µg/cm^2	Yellow	Lime green	Y	

Live Agent testing at NATO Project Group 31 meeting at the Centre d'Etudes du Bouchet (CEB) Test Facility, Cazaux, France

The NATO Project Group 31 (PG/31), which deals with the development of "Non-Corrosive, Biotechnology-Based Decontaminants for CBW Agents" held its sixteenth meeting on 11-16 Sep 2002 at the Centre d'Etudes du Bouchet (CEB) Test Facility,

Cazaux, FR. PG/31 consists of six member nations: France (FR), Germany (GE), Italy, (IT), Turkey (TU), the United Kingdom (UK) and the United States (US). As part of the meeting, Agentase Traffic Light sensors were demonstrated during various live agent tests.

Figure 30. French soldier activating Agentase TLS on table (left) and sensors after testing yielding yellow, green and red colors (right)

The following sensors underwent green color development in 15 minutes or less:

- Test plates with no applied agent
- Sensors dipped into the German enzyme solution for nerve agent decon (consists of enzymes, fire fighting foam surfactants (ECHO-foam), and buffer)
- Test plates sprayed with German enzyme-foam decontaminant

Red color was observed in less than 1 minute with the following samples:

- 10g/m^2 GD (Soman) - NATO standard for contaminated surfaces
- 1.0g/m^2 GD
- 0.1g/m^2 GD (representing 99% decon of nato std contamination levels - STILL REPRESENTS A CONTACT HAZZARD
- Identically contaminated plates were sprayed with the German decon solution NOT CONTAINING ANY ACTIVE INGREDIENT (enzyme)
  Each plate still gave a positive response (Extraction and analysis using GD showed that the 100cm2 plates still carried between 30-60ug of agent - previous data suggests that Agentase sensors can detect as little as 1 ug of agent on a plate.)

Due to the successful demonstration various members expressed interest upon testing the Agentase TLS and Training sensor. The Agentase product was featured in a technology demonstration provided to NATO representatives. The following text is taken from the minutes record resulting from the NATO meeting:

"For the Traffic Light Sensor, positive responses were observed at the three levels of contamination used: 10, 1, and 0.1 g/m^2 (corresponding to 100, 10, and 1 mg of agent per plate). A number of participants expressed interest in learning more about this technology."

Joseph DeFrank, Chairman NATO PG 31 Group

Meeting minutes - 16th meeting, Septmber 2002.

Live agent testing at DSTL Porton Down, 2nd of April, 2003

Agentase arranged a testing of the Yellow-Red sensor version at DSTL in Porton Down.

"The sensors are supplied in a package which would be easy to open whilst wearing full IPE. The design of the sensors allows activation on a clean surface (provision of yellow dish). Every sensor supplied for use in this test activated effectively. There was no sign of leakage during transport. The operator would be able to use the sensor without him/her contacting the sensor sponge before or after the swabbing process whilst wearing full IPE, and so avoid the risk of contamination. The colour change of the sensors is not a permanent effect, and in many cases they revert to a yellow colour after 16 hours. (These observations were made out of idle curiosity, and as such have not been included in the table of results). The sensors appear to be much more responsive to the presence of GD than any of the other agents, and can detect it with certainty down to 4ug/ml. VX appears to be the most difficult to detect at lower concentrations. The clear Orange colour can not be mistaken for a negative response, and is likely to show up as a clear positive response even when the sensor is wiped over a dirty surface (muddy, dusty etc.) This would suggest that the lowest concentration of agent, which would result in an unambiguous response from the sensors, irrespective of the agent detected, is 8 to 10ug/ml. At concentrations lower than that, the sensor responses begin to vary depending on the agent being used."

Shelley Lowe, DSTL Porton Down, UK April 2nd, 2003

Table 4. Live agent data from DSTL utilizing the Yellow-red version

Agent	Nominal Concentration (ug/ml)	Actual Concentration (ug/ml)	Temperature of test	Sensor Colour at 2 minutes	Sensor Colour at 25 mi	Response
GA	100	116	23.5	Pink / Red	Vibrant Pink / Red	Positive
GA	100	136	23.6	Pink / Red	Vibrant Pink / Red	Positive
GB	100	101	24.2	Orange / Pink	Vibrant Pink / Red	Positive
GB	100	93	23.8	Orange / Pink	Vibrant Pink / Red	Positive
GD	100	160	23.6	Pale Pink / Red	Vibrant Pink / Red	Positive
GD	100	143	23.9	Pale Pink / Red	Vibrant Pink / Red	Positive
VX	100	122	23.9	Pale Pink / Red	Vibrant Pink / Red	Positive
VX	100	118	23.6	Pale Pink / Red	Vibrant Pink / Red	Positive
GA	20	26	24.4	Pale Pink / Red	Pink / Red	Positive
GA	20	27	24.5	Pale Pink / Red	Pink / Red	Positive
GB	20	30.6	24.7	Pale Pink / Red	Pink / Red	Positive
GB	20	32.6	24.6	Pale Pink / Red	Pink / Red	Positive
GD	20	30.2	23.6	Pale Pink / Red	Vibrant Pink / Red	Positive
GD	20	29	23.7	Pale Pink / Red	Vibrant Pink / Red	Positive
VX	20	25.8	24.4	Orange / Pink	Pink / Red	Positive
VX	20	25.6	23.9	Orange / Pink	Pink / Red	Positive
GA	8	9.6	24.5	Orange / Pink	Orange / Pink	Positive
GA	8	8.08	23.7	Orange / Pink	Orange	Positive
GB	8	7.92	23.7	Pale Orange	Pink / Red	Positive
GB	8	8.4	23.6	Pale Orange	Orange / Pink	Positive
GD	8	10.4	21.9	Pale Orange	Vibrant Pink / Red	Positive
GD	8	12	21.8	Orange / Pink	Vibrant Pink / Red	Positive
VX	8	10.48	21.6	Darker Yellow	Orange	Positive
VX	8	10.8	21.4	Darker Yellow	Orange	Positive
GA	4	5.92	21.9	Pale Orange	Orange	Positive
GA	4	5.72	21.8	Darker Yellow	Orange / Pink	Positive
GB	4	5.36	21.6	Pale Orange	Orange / Pink	Positive
GB	4	5.08	21.9	Pale Orange	Orange / Pink	Positive
GD	4	5.44	22.7	Pale Orange	Pink / Red	Positive
GD	4	5.92	23.3	Pale Orange	Pale Pink / Red	Positive
VX	4	4.92	22.6	Yellow	Pale orange	Negative
VX	4	5.4	23.4	Yellow	Darker Yellow	Negative
GA	1	1.34	23.3	Yellow	Yellow	Negative
GA	1	1.27	23.4	Yellow	Yellow	Negative
GB	1	1.12	23.5	Yellow	Yellow	Negative
GB	1	1.15	23.5	Yellow	Yellow	Negative
GD	1	1.15	23.8	Darker Yellow	Pale orange	Negative
GD	1	1.16	23.7	Darker Yellow	Pale orange	Negative
VX	1	1.16	23.6	Yellow	Yellow	Negative
VX	1	0.75 *	23.9	Yellow	Yellow	Negative
GA	0.2	0.12 *	22.9	Yellow	Yellow	Negative
GA	0.2	0.10 *	23.2	Yellow	Yellow	Negative
GB	0.2	0.20	23.2	Yellow	Yellow	Negative
GB	0.2	0.28	23.0	Yellow	Yellow	Negative
GD	0.2	0.23	23.8	Yellow	Yellow	Negative
GD	0.2	0.19	23.7	Yellow	Yellow	Negative
VX	0.2	0.30	23.5	Yellow	Yellow	Negative
VX	0.2	0.28	23.6	Yellow	Yellow	Negative

This data not only shows that the Agentase Traffic Light sensor and the Yellow-Red sensor version are useful in detecting ng/cm^2 levels of nerve agents, but more importantly also demonstrates that simulant data from Agentase's laboratory correlates well with live agent data conducted by independent third parties. Further tests are arranged. These tests include a comparison between the TLS and the water test sensor.

Production

The current polymer synthesis method yields either 150 substrate disks or 50 enzyme disks, since the enzyme disks are about 3 times thicker than the substrate disks. Immediately after synthesis the bulk enzyme polymers need to be cut into slices using a cutting tool which has been designed and constructed by Agentase. The bulk substrate polymers need to be placed into a –70^{o}C freezer for about one hour prior to cutting. The freezing step is necessary since the soft polymer foam is incompatible with the commercial cutting machine we utilize in cutting the bulk polymer into ¼ inch slices. Seven individual disks can be cut out of each slice using a 20-ton press with custom-made dies (see Figure 31). The die for the enzyme polymer includes crosses within each circle to create a cross cut which accommodates the red valve of the sensor. The whole preparation of polymer disks takes place one day prior to sensor assembly since the moist polymers need to be air dried overnight to optimize performance.

Figure 31. Enzyme polymer slice before and after cutting out of individual disks

Prior to assembly of the Agentase TLS the enzyme disks need to be attached to the valves by melting two ridges at the top of the valve base. For this process a standard laboratory hotplate is sufficient. A cross cut within the polymer accommodates the red tip of the valve, which is not visible from the outside of the assembled sensor. The reservoir needs to be filled with water prior plugging in the polymer-valve assembly and

securing it with a nylon ring. As a final step, the reservoir needs to be placed into the black base and the finished sensor can be then sealed with a clear cap. The sensor and substrate cap are packaged within a mylar pouch for protection from the outside environment and to maximize shelf-life. The whole assembly and packaging process takes about 2 minutes per sensor.

One person is easily able to assemble 30 sensors per hour. Assuming 2 hours downtime per day and 1 hour to produce the required polymers this *one person can easily produce 150 sensors per day or 450 sensors per work week*. While the assembly process does not require any expensive equipment this process is easy to scale up by utilizing more people. The bottleneck can be seen within the polymer synthesis since this requires more expensive equipment. Assuming a six-hour production, one person can produce polymer disks for 900 sensors. The assembly of 900 sensors per day requires 5 additional people. Therefore *six people can assemble 900 sensors per day, or 4500sensors per work week* utilizing the current production equipment of Agentase without any significant investments in additional machinery. To test these numbers, Agentase assembled once 400 sensor units on one single day using three people.

These numbers clearly show that Agentase possesses the capacity to produce 500 sensors a day, 2500 sensors per week using the currently available resources and personnel. After hiring more production personnel this capacity can be easily scaled up to 900 sensors a day or 4500 sensors per week.

Quality control

The quality control is an important aspect of any production process. There are in principal two solutions feasible to verify the functionality of the TLS: a) Measure the enzymatic activity of each enzyme using independent assays, b) Measure optically the colorimetric signal response of the entire sensor. While the enzymatic method measures the quality of the enzyme polymer, only the optical method verifies the performance of the entire sensor. Therefore we decided to use the optical method as first choice in testing the quality of individual sensor batches.

Conclusion

This final progress report clearly shows that Agentase succeeded in its task in developing its nerve agent sensor from initial proof-of-concept to a commercial and field tested product. Agentase clearly achieved its goal in developing "*a simple-to-use enzyme-containing sensor for detecting nerve agent contamination at surfaces, in air and in solution, and to provide a tool for early and accurate identification of the chemical agents*". End user feedback shows that the Agentase sensor is well accepted. This report demonstrates that Agentase has meet its deliverables:

- User community beta-testing
- Refine sensor platform based on beta-testing results
- Prototype sensor interference testing
- Proof-of-Concept/Feasibility: Non-point source applications
- Pre-production sensor release

In addition Agentase will send 500 of its final sensors to DTRA for further product evaluation and testing.

List of all publications and technical reports supported under this contract

Papers presented at meetings, but not published in conference proceedings

- Erbeldinger, M. The use of the Agentase Traffic Light Sensor, Defense Intelligence Agency, Washington DC, April 2001.
- LeJeune, K.E. Traffic Light Sensor for nerve agent detection, Onsite conference, San Diego, CA, January 2002.
- LeJeune, K.E. Traffic Light Sensor for nerve agent detection, NATO Project Group 31 meeting at the Centre d'Etudes du Bouchet (CEB) Test Facility, Cazaux, France, September 2002
- LeJeune, K.E. Traffic Light Sensor for nerve agent detection, Department of Defense Day on the Hill, Washington DC, July 2002.
- Erbeldinger, M. and LeJeune, K.E. CW-related enzyme-polymer product development at Agentase and its use as an End of Service Life Indicator, End of Service Life Indicator (ESLI) conference Panama City, FL, October 2002.
- LeJeune, K.E. Traffic Light Sensor for nerve agent detection, Meeting with BG Nilo and LTC Serino at the US Army Chemical School at Fort Leonard Wood, MO, October 2002.
- LeJeune, K.E. Traffic Light Sensor for nerve agent detection, Meeting with Deputy Assistant to Secretary of Defense Ana Johnson-Winegar, Pentagon, Washington DC, December 2002.
- LeJeune, K.E. Traffic Light Sensor for nerve agent detection, DARPA Transition of Biodefense Technology Program Meeting, Arlington, VA, January 2003.
- LeJeune, K.E. Traffic Light Sensor for nerve agent detection, DTRA Program Meeting, Arlington, VA, February 2003.

Technical reports submitted to ARO

- LeJeune, K.E. Nerve Agent Sensing Biopolymer Wipe (Quarterly Technical Report I), Remove requirement for applied developing solution, July 2001.
- LeJeune, K.E. Nerve Agent Sensing Biopolymer Wipe (Quarterly Technical Report II) Optimize sensor formulation for maximum performance, October 2001.
- LeJeune, K.E. Nerve Agent Sensing Biopolymer Wipe (Quarterly Technical Report III), Address potential environmental sensitivity of sensor, January 2002.
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- LeJeune, K.E. Nerve Agent Sensing Biopolymer Wipe (Quarterly Technical Report V) Continuing prototype testing and development, July 2002.
- LeJeune, K.E. Nerve Agent Sensing Biopolymer Wipe (Quarterly Technical Report VI) Independent testing of sensor and accompanying materials, October 2002.
- LeJeune, K.E. Nerve Agent Sensing Biopolymer Wipe (Quarterly Technical Report VII) Finalize sensor "production", January 2003.

List of all participating scientific personnel showing any advanced degrees earned by them while employed on the project

Not applicable

List of all participating scientific personnel

- Allinson, Bryan
- Erbeldinger, Markus Ph.D.
- Heinbaugh, Danielle
- LeJeune, Keith Ph.D.
- Mysliwczyk, Richard
- Proch, Melissa
- Williams, Cindy

Report of Inventions

- LeJeune, K.E., Erbeldinger, M., Positive Response Biosensors and Other Sensors, US Patent application, Submitted May 2001.
- LeJeune, K.E., Erbeldinger, M., Sensors for the Detection of an Analyte, US Patent application, Submitted Nov 2001.

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